ON THE INFLUENCE OF ALLOSTERIC EFFECTORS ON THE ELECTRON PARAMAGNETIC SPECTRUM OF NITRIC OXIDE HEMOGLOBIN

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1. Introduction

Organic polyphosphates such as ATP and 2,3-diphosphoglycerate are known to lower the oxygen affinity of hemoglobin [1], the most pronounced effect being achieved with inositol hexaphosphate (IHP) [2]. The binding site of 2,3-diphosphoglycerate is formed by cationic groups of amino acids of both β-chains within the central cavity of the hemoglobin tetramer [3]. This binding site has also been discussed for IHP [4]. Organic polyphosphates induce conformational changes of the protein, thereby weakening the iron ligand field and reducing the ligand affinity of the heme iron [5]. To examine the changes of the electronic structure of the iron-bound ligands by polyphosphates directly, we investigated the paramagnetic nitric oxide hemoglobin (NO–Hb) using the electron paramagnetic resonance method (EPR).

2. Methods

NO–Hb was prepared from fresh Hb-A according to a method described earlier [6]. EPR measurements were performed with an X-band superheterodyn spectrometer [7] at a temperature of 77°K. The spectra were recorded as the first derivative of the resonance absorptions. The intensity of the hyperfine structure was determined by integration of the hyperfine lines relative to a base line which halves the hyperfine line. This value was correlated with the overmodulated signal of NO–Hb.

3. Results

The EPR spectrum of NO–Hb is characterized by a relatively broad anisotropic line of approximately axial symmetry with g = 2.066 and g = 2.00 (fig. 1A). At neutral pH allosteric effectors such as IHP bring about a hyperfine structure with three lines (fig. 1B) which originate, according to Kon [8], from the interaction of the unpaired electron of the NO group with the nitrogen nucleus of this group on account of their number and the distance between them (16.5 Gauss). The intensity of the hyperfine structure depends on the concentration of the effector (fig. 2). In this figure the signal intensity is plotted versus the total concentration of the effector and therefore the plot is not hyperbolic. By means of a mathematical simulation based on the law of mass action the binding constant has been estimated to be 10^5 to 10^6 M^{-1}. The binding capacity suggests a stoichiometry of one mole IHP per mole hemoglobin.

ATP and 2,3-diphosphoglycerate produce the same kind of hyperfine structure, but higher molar concentrations of these allosteric effectors are necessary to produce the hyperfine structure. At a hemoglobin concentration of 8×10^{-4} M the maximum effect is achieved with 10^{-2} M ATP or 2,3-diphosphoglycerate, because these effectors have about a 10-fold lower affinity than IHP.

Also the lowering of pH produces the same hyperfine structure in the EPR spectrum of NO–Hb. Fig. 3 demonstrates the increasing intensity of the hyperfine structure with increasing H^+ concentration in the absence of allosteric effectors. Because of denaturation

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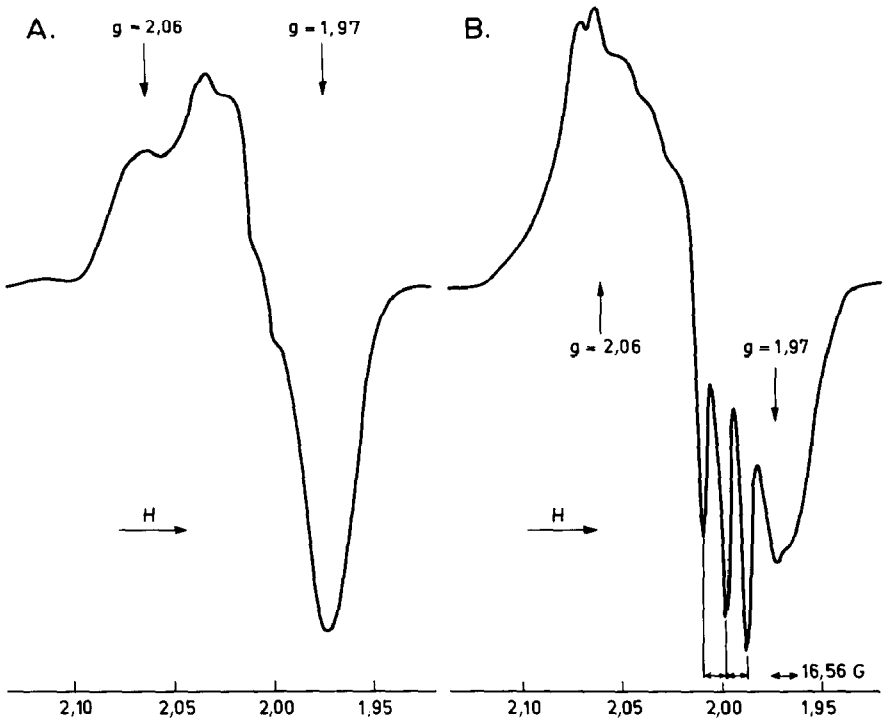

Fig. 1. A) EPR spectrum of NO–Hb; B) with 5×10^{-4} M IHP; pH 6.9; 0.2 M Tris buffer; Hb-concentration: 8×10^{-4} M as heme.

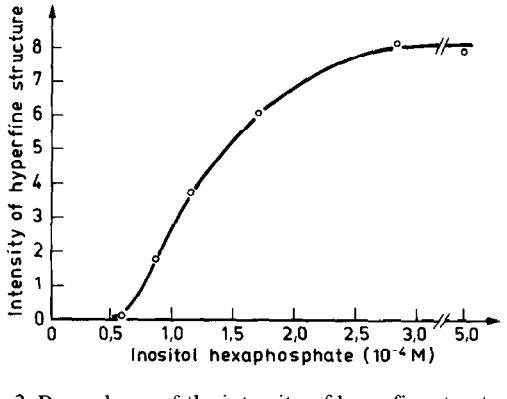

Fig. 2. Dependence of the intensity of hyperfine structure in the EPR spectrum of NO–Hb on concentration of IHP; conditions as in fig. 1.

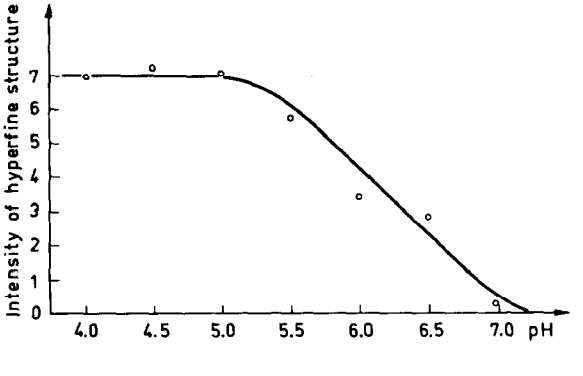

Fig. 3. Dependence of the intensity of hyperfine structure in the EPR spectrum of NO–Hb on pH; the pH was adjusted by addition of 0.1 M HCl; Hb-concentration: 8×10^{-4} M as heme.

of the protein, the hyperfine structure is no longer detectable below pH 4. The hyperfine structure observed at pH 4.5 to 5.0 reversibly disappeared as the medium was neutralized. At alkaline pH (> 8), even in the presence of effectors, no hyperfine structure is detectable. High concentrations of NaCl (0.3 M) failed to produce a hyperfine structure.

The action of allosteric effectors on the EPR spectrum of NO–Hb depends on pH. In alkaline medium higher effector concentrations are necessary to produce the hyperfine structure than in acidic

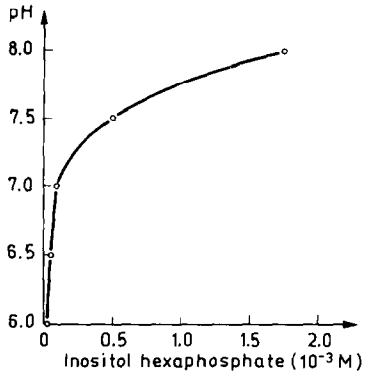

Fig. 4. Dependence of pH on the concentration of IHP, which produces half the intensity of the hyperfine structure.

medium. Fig. 4 shows that with increasing pH the concentration of IHP must be higher to produce the half intensity of the hyperfine structure. From this dependence it can be concluded that an increasing protonation of hemoglobin produces a conformational change with the same effect on the spin label (NO group) as the allosteric effectors.

4. Discussion

EPR studies of NO heme nitrogenous base complexes by Kon [9] showed the hyperfine splitting constant to depend on the kind of base. In our study no changes of the hyperfine splitting constant were observed, but a change of the hyperfine structure intensity depending both on effector concentration and pH. Consequently, the influence exerted by the allosteric effectors or by pH upon the electronic structure of the NO–iron bond is not only a change of the electronic structure of the proximal histidine (F8). Changes of the intensity of the hyperfine structure reflect first of all a change in the binding geometry of the ligand which is enforced by changes occurring in the spatial structure in close vicinity of the ligand.

In NO–Hb, NO is bound to the iron at an angle of 110° [10]. In this bond $d\pi-p\pi$ electron back-bonding is involved. A slight turn of the NO group around the heme axis will weaken the π-bond of NO with the d_xz or d_yz orbital of the iron. Mössbauer measurements have revealed a strong spin transfer for NO–Hb [11]. This spin transfer is diminished by reduction of the π-bond which alters the spin density at the nitrogen nucleus in such a manner that a hyperfine structure appears. NO–Hb and oxy-Hb are assumed to have similar electronic structures [12] as well as equal spatial structures. X-ray studies by Watson and Nobbs [13] have shown that the oxygen in oxymyoglobin is bound angularly. If we assume, in accordance with the ideas of Pauling [14], that in hemoglobin also the oxygen is bound angularly, then the change of the electronic structure of NO–Hb induced by allosteric effectors would mean the reduction of binding affinity to the ligand not only for NO–Hb, but also for oxy-Hb.

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